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Stable isotope composition to validate ecological network models: the case of Arrábida Marine Park rocky reefs

Helena Cristina Rosa Pires

Mestrado em Ecologia Marinha

Dissertação orientada por:
Doutora Susanne Tanner
Doutora Sofia Henriques

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Abstract

Coastal marine habitats provide important environmental and socio-economic services, and adjacent coastal areas are very attractive to human populations. Like other coastal marine habitats, rocky reefs are heavily impacted by anthropogenic activity. As these are considered important breeding, nursery and feeding areas for several marine species, their protection is imperative. To improve conservation methods, it is necessary to understand the structure and processes of rocky reef communities. Trophic relationships are one of the ways in which species are connected, with food webs representing the flow of matter and energy from producers to consumers and from prey to predators. Stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) was used to characterize the food web of the rocky reef of Arrábida Marine Protected Area. The results showed a relatively short food web, most likely due to high abundances of juveniles with consumers with diverse diets and feeding strategies, exploiting different sources of organic matter, of both benthic (macroalgae and benthic POM) and pelagic origin (phytoplankton and pelagic POM). However, the benthic pathway was more important with more than half of the diet of most secondary and tertiary consumers following this route and thus, suggesting a bottom-up control, and hinting at the importance held by macroalgae and benthic production in this ecosystem. There was also a high incidence of omnivores in all trophic groups, which could contribute to the similar trophic redundancy and trophic evenness found between groups. Even so, primary consumers showed greater trophic richness related to basal resources.

Key-words: Temperate rocky reef; food web; stable isotopes; trophic level; trophic pathways

Resumo

Os habitats marinhos costeiros fornecem importantes serviços ambientais e socioeconómicos, sendo as zonas costeiras adjacentes muito atrativas para as populações humanas. Tal como os outros habitats marinhos costeiros, os recifes rochosos são bastante impactados pela atividade antropogénica. Sendo estes considerados zonas importantes de reprodução, berçário e de alimentação para várias espécies marinhas, torna-se imperativa a sua proteção. Para melhorar os métodos de conservação é necessário compreender a estrutura e os processos das comunidades dos recifes rochosos. As relações tróficas são uma das formas pelas quais as espécies estão conectadas, sendo as teias alimentares representações do fluxo de matéria e energia dos produtores aos consumidores e das presas para os predadores. A análise de isótopos estáveis ($\delta^{13}C$ e $\delta^{15}N$) foi usada para caracterizar a teia trófica do recife rochoso da Área Marinha Protegida (AMP) da Arrábida. Os resultados mostraram uma teia alimentar relativamente curta, provavelmente devido à maior abundância de juvenis, com consumidores com dietas e estratégias alimentares diversificadas, explorando diferentes fontes de matéria orgânica, tanto de origem bentónica (macroalgas e POM bentónico) como de origem pelágica (fitoplâncton e POM pelágico). No entanto, a via bentónica contribuiu em mais da metade para a dieta da maioria dos consumidores secundários e terciários, sugerindo um efeito *bottom-up* e mostrando a importância das macroalgas e da produção bentónica neste ecossistema. Houve também uma alta incidência de omnívoros em todos os grupos tróficos, o que pode contribuir para a similar redundância trófica e uniformidade trófica encontrada entre os grupos. Contudo, os consumidores primários apresentaram maior riqueza trófica relacionada aos recursos basais.

Palavras-chave: Recife rochoso temperado; teia alimentar; isótopos estáveis; nível trófico; vias tróficas

Resumo alargado

Os habitats costeiros são essenciais para a subsistência humana, fornecendo bens e serviços importantes, como o ciclo de nutrientes, alimento e matéria-prima. Por isso mesmo, são também os mais afetados por pressões antropogénicas, que ameaçam a estrutura funcional dos sistemas marinhos. Dentro dos habitats costeiros, os recifes rochosos são uns dos mais afetados. Este habitat é caracterizado por uma elevada complexidade arquitetural e pela cobertura de algas, que são elementos fundamentais para a sua elevada biodiversidade e variabilidade espácio-temporal, exibindo comunidades complexas compostas por grande parte dos grupos taxonómicos. Os recifes rochosos são também importantes zonas de alimentação e berçário, promovendo a sobrevivência e o crescimento de juvenis. Tendo isto em conta, a proteção deste habitat torna-se uma prioridade nos esforços de conservação.

Para melhorar estratégias de gestão e conservação dos ecossistemas é necessário ter uma compreensão profunda dos mesmos, principalmente referente à sua suscetibilidade a perturbações e à extinção de espécies. Como tal, o estudo de redes ecológicas tem sido essencial para entender as dinâmicas e a estabilidade da biodiversidade. As teias tróficas são um tipo de redes ecológicas que representam as relações alimentares dentro de uma comunidade e correspondem aos fluxos de energia e nutrientes entre os organismos. A análise de isótopos estáveis é um dos métodos mais comuns para analisar teias tróficas, uma vez que permite estimar as relações tróficas entre espécies, calcular os seus níveis tróficos, o comprimento das cadeias alimentares e a contribuição relativas das fontes alimentares. O carbono (C) e o azoto (N) são os elementos mais usados nos estudos de isótopos estáveis em teias tróficas marinhas. O rácio de δ^{13}C pode ser usado para determinar a origem da matéria orgânica e a sua via trófica; o rácio de δ^{15}N torna possível determinar a posição trófica e a topologia básica da teia trófica.

Este estudo tem como objetivo caracterizar a teia trófica do recife rochoso do parque marinho Professor Luiz Saldanha, Área Marinha Protegida (AMP) da Arrábida, analisando os isótopos estáveis das espécies e grupos de espécies mais representativos deste ecossistema, de forma a perceber a estrutura e os processos ecológicos desta teia alimentar.

A AMP da Arrábida é composta por áreas com diferentes níveis de proteção, com zonas de Proteção Total, com uma política *no-take*, onde as atividades humanas não são permitidas, zonas de Proteção Parcial, onde a pesca com instrumentos específicos é permitida a 200 m da costa, e zonas de Proteção Complementar, onde algumas atividades de pesca são permitidas e há mais pressão humana. Para além de um gradiente de pressão antropogénica este parque marinho tem um gradiente ambiental causado pelo estuário do rio Sado. Neste estudo, a amostragem foi realizada entre outubro e janeiro, na zona de proteção total, por ser considerada a linha de base deste ecossistema. Foram realizadas colheitas em mergulho entre os 5 m e os 12 m de profundidade, com redes, armadilhas e raspagens e recolhas de fitoplâncton, zooplâncton e POM pelágico com redes de arrasto e garrafas de Van Dorn. Em laboratório, as amostras foram secas e trituradas num pó fino. Depois de processadas em laboratório, os rácios ^{13}C/^{12}C e ^{15}N/^{14}N das amostras foram determinados por espectrometria de massa de razões isotópicas de fluxo contínuo. Os dados foram representados num gráfico δ^{13}C/ δ^{15}N, e foram calculadas métricas para descrever a comunidade (métricas de Layman), a posição trófica e a contribuição bentónica para a dieta dos organismos, utilizando as amostras de zooplâncton e Polyplacophora como *proxy* da via pelágica e bentónica, respetivamente.

Neste estudo, as amostras dos consumidores superiores (secundários e terciários) eram compostas principalmente por juvenis e indivíduos de menor tamanho. Isto era esperado, tendo em conta que os recifes rochosos são importantes áreas de berçário. Consequentemente, estes organismos apresentaram posições tróficas inferiores ao que está comumente descrito na bibliografia, uma vez que

a sua dieta varia com a ontogenia. Por sua vez, o comprimento máximo da cadeia alimentar foi relativamente curto comparado com outros ecossistemas marinhos, mas similar a outros recifes rochosos. É também possível que haja uma grande abundância de omnívoros nesta teia trófica, o que pode explicar também a semelhante redundância trófica encontrada entre os três grupos tróficos (consumidores primários, secundários e terciários). Os resultados mostraram uma grande amplitude dos valores de δ^{13}C nos produtores e consumidores primários, indicando que a teia alimentar do recife rochoso da MPA da Arrábida tem por base uma grande diversidade de fontes alimentares. Os consumidores primários e secundários apresentaram também nichos tróficos maiores, podendo indicar uma maior diversidade trófica nestes grupos.

Nesta teia trófica pode-se identificar duas vias tróficas, pelágica e bentónica. A via pelágica tem como fontes o fitoplâncton e o POM (matéria orgânica particulada) pelágico. Por sua vez, a via bentónica corresponde às macroalgas e ao POM bentónico. Os resultados mostraram que ambas as vias contribuem para a dieta dos consumidores analisados e consequentemente para a teia trófica deste recife rochoso. A via pelágica foi o maior contribuidor para alguns consumidores, tal como os misidáceos, *Felimare tricolor*, o bodião-rupestre (*Ctenolabrus rupestris*) e o góbio-nadador (*Pomatoschistus flavescens*). O fitoplâncton e o POM pelágico são importantes fontes para os filtradores, que por sua vez, ao expelirem nutrientes e serem consumidos por organismos bentónicos e demersais, fazem uma ligação entre os sistemas pelágico e bentónico. Por outro lado, a contribuição bentónica foi superior para todos os outros consumidores, com as macroalgas e o POM bentónico a ter uma importante contribuição para a dieta dos consumidores desta teia trófica.

As macroalgas são especialmente importantes pois não só são importantes fontes alimentares, mas também contribuem para a complexidade estrutural do recife, fornecendo abrigo a predação, e zonas de berçário. Nos recifes rochosos as macroalgas, em particular os kelps, estão associadas a cascadas tróficas, evolvendo ouriços-do-mar e os seus predadores. Neste estudo, a macroalga da família dos kelps, *Saccorhiza polyschides*, aparenta ser a maior contribuidora para a dieta dos ouriços-do-mar *Sphaerechinus granularis*, e em conjunto com outro estudo feito neste parque marinho, no qual a possível diminuição dos predadores de *Sphaerechinus granularis*, como por exemplo o *Diplodus vulgaris*, *Diplodus sargus* e o *Coris julis*, significou um aumento na abundância de ouriços-do-mar e uma diminuição da cobertura destas algas, há evidência que esta interação trófica ocorre. O POM bentónico é também importante para esta teia trófica, sendo incorporado por detritívoros. O POM bentónico apresentou valores de δ^{13}C significativamente superiores aos valores dos outros produtores primários analisados, estando mais próximo dos valores do fitoplâncton. É possível que o fitoplâncton contribua para a composição do POM e que este tenha ficado enriquecido em δ^{13}C com a degradação das partículas no fundo.

Os recifes rochosos temperados são altamente sazonais e o parque marinho da Arrábida é adicionalmente afetado por eventos de afloramento costeiro. Para além disso existe também um forte gradiente espacial, tanto na cobertura de algas, como nas pressões antropogénicas. Tendo isto em conta, para entender completamente a estrutura e as dinâmicas desta rede trófica são necessários estudos adicionais, incluindo a variação sazonal e espacial, e organismos representantes de todas as fases de vida. Este estudo é um passo importante para perceber os processos inerentes às teias tróficas dos recifes rochosos temperados e, em conjunto com outros estudos, é uma ferramenta importante para melhor prever as respostas do ecossistema da AMP da Arrábida a ameaças humanas e a alterações ambientais.

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General Introduction

Food webs represent feeding relationships within a community, they are networks of consumer-resource interactions and correspond to flows of energy and nutrients between a group of organisms or populations (Layman et al., 2015). Food web research has increased its focus to what food webs can reveal, from ecosystem susceptibility to species extinction, with the objective of improving ecosystem management strategies (McDonald-Madden et al., 2016). But despite its importance, there is no single theory or set of methods for studying food webs. In fact, there is a wide array of methodological approaches, from empirical studies (e.g. Heijboer et al., 2017; Traugott et al., 2013) to mathematical theory (e.g. Cohen et al., 1990; Cozzens, 2015) to sophisticated ecological network analysis (e.g. D'Alelio et al., 2016; Dunne et al., 2002)

Early food web diagrams were the interconnection of the known trophic chains to one another and were based solely on the feeding relations known at the time. Even so, they were of vital importance to unravel the direct and indirect interdependence of organisms (Elton, 1927 in Zanden et al., 2016). For many, the birth of Food Web Ecology is associated with Charles Elton and his work Animal Ecology, published in 1927. In it, Elton discusses food chains and food cycles, i.e. the total sum of all food chains in a system, and its importance for understanding the functioning of ecosystems. According to him, the fact that predators tend to be larger than their prey is an intrinsic property of ecological systems and is important for the structure of animal communities. Elton also introduced the idea of number pyramids, where primary producers and herbivores form the base of food webs, as they are typically more abundant and smaller. Furthermore, Elton emphasized the niche as an animal's functional role in the ecosystem, especially regarding what it eats. Finally, he noted that one species can influence another, through the mediation of more intervening species, demonstrating the complexity of interspecific interactions (see Layman et al., 2015 for comprehensive historical review of food web ecology).

Early studies of food webs describe relatively static interactions between organisms, which fluctuated around an equilibrium (Zanden et al., 2016). Nowadays, it is accepted that food webs are dynamic, both in the type of interactions and in the consequences that these interactions have on organisms. Instead of focusing on direct interactions between species, namely the predator-prey relationship, it is possible to interpret the interactions as flow of energy (Lindeman, 1942 in Zanden et al., 2016). Thus, the concept of trophic dynamics emerges, describing the position that an organism occupies in an ecosystem, that its, its trophic level (Moreno-Sánchez et al., 2016), based on the transference of energy from one part of the ecosystem to another (Lindeman, 1942 in Layman et al., 2015). The main topic related to trophic dynamics is related to the indirect effects caused by interactions between organisms, which control and regulate production and resources. For example, when a trophic level in a food web is suppressed, trophic cascades occur (Layman et al., 2015). A trophic cascade is therefore a dynamic interaction where the effects of predatory feeding on prey propagate down more than one link in a food web, causing changes to the structure of an ecosystem (Pace, 2013). The most commonly known types of forcing are bottom-up and top-down, but subsidy cascades, where native species feed on resources that do not originate in their habitat, can also occur (Luskin et al., 2017; Zhang et al., 2003). It was first observed that the energy was produced through photosynthesis, converted into plant biomass, consumed by herbivores, and transformed into herbivore biomass and so on throughout the food chain (Lindeman, 1942 in Zanden et al., 2016). Production decreased in successive trophic levels since metabolic reactions are not 100% efficient. In this way, primary production was the limiting factor for production at higher trophic levels, suggesting a bottom-up control of the distribution of biomass (Pimm, 1982; Power, 1992 in Zanden et al., 2016). This realization marked a significant advance in Ecology, as it provided an operational framework for the investigation of food webs by

introducing the concept of trophic levels and the use of energy as currency (Layman et al., 2015). Later, it was recognized that top-down control was also possible, as predators control the population size of herbivores, allowing the accumulation of plant biomass (Hairston, 1960 in Zanden et al., 2016). This way, the effect that predators have on the abundance of their prey cascades down the trophic chains and impacts primary producers. To demonstrate the dominance of top-down factors, removing predators from a three-tier system should increase herbivore biomass and decrease plant biomass. If the opposite occurs, this indicates a bottom-up control (Zanden et al., 2016). Currently, it is thought that both "top-down" (Asnaghi et al., 2013; Guidetti, 2007) and "bottom-up" forces can influence community structure on rocky reefs (Azzurro et al., 2013; Hereu et al., 2008; Miller & Etter, 2011).

Food webs can be highly complex, with hundreds of species and trophic links, omnivore relationships, and predation. A food web can not only refer to the energy flow but also to the demographic control that one species has on another (Layman et al., 2015). These food webs are called functional webs and differ significantly from those based on energy transfer. (Layman et al., 2015). In this way, food webs can be of three types: topological, energy, or functional. Topological food webs, also known as connectedness food webs, are represented by simple diagrams with boxes representing species or groups of species and arrows representing trophic interactions between them or resource uptake, emphasizing feeding relationships (Moore & De Ruiter, 2012a). However, this type of food web is based on a binary system, depicting only the presence or absence of a trophic interaction, and does not show the strength of said interaction. Energy flow webs quantify energy flow from one species to another, making it possible to depict the strength of the relationship. Energy flow or energy flux webs provide quantitative data about the distribution and transfer of energy and matter within the community, making it possible to estimate rates of mineralization, such as inorganic carbon respiration and nitrogen excretion, in the whole food web or in individual functional groups (Moore & De Ruiter, 2012b). Functional food webs intend to identify species that have strong effects on population dynamics (Moore & De Ruiter, 2012c). This type of food web aims to depict the influence of different populations in the growth rate of other populations and emphasizes the importance of each species in maintaining the integrity of a community (Paine, 1980 in Hui, 2012). For this type of food web, the interaction strength is what defines the links between species or groups of species. There are several methods to quantify the strength of trophic interactions, such population models and even energy flow webs (Moore & De Ruiter, 2012c). Although energy and functional webs are often confused, they are not necessarily equivalent since a strong link, quantified by the energy flow, does not imply a strong control of a consumer species in the population size or in the growth rate of its particular prey (Layman et al., 2015).

The term "food web structure" can have several meanings (Zanden et al., 2016). It can refer only to the number of trophic levels in a food chain, or it may represent a food web linkage network. Diagrams of food webs are used to represent the energy flow pathways throughout the system, or, alternatively, the linkages that are dynamically important for the regulation of the abundance of other organisms. Additionally, the structure of the food web can refer to the distribution of biomass through the trophic levels (Zanden et al., 2016). In food webs, the links represent the food connections in the ecological community, with the number of links per consumer designated as connectance (Dunne et al., 2002).

It became evident the need to quantitatively detail the structural properties of food webs using metrics to characterize them (Layman et al., 2015). Several metrics can give an insight into the dynamics and biomass distribution and production in an ecosystem. Among these metrics, there is the specific richness, the number of interactions between species or links, and many more properties and emerging statistics derived from these metrics (Kortsch et al., 2019; Link, 2002; Link et al., 2012). Examining these metrics can be useful for comparing ecosystems with food webs constructed in the same way

(Dunne et al., 2002). With the advancement in network modelling, the Network Theory emerged, which sees food webs within a mathematical perspective (Dunne et al., 2002).

Food webs are complex in the way that they change in scale, seasonally, and geographically (Dunne et al., 2002). This is especially true in marine ecosystems, as there are many organisms that travel great distances. Even nutrients cross the thresholds of ecosystem boundaries. This environmental heterogeneity causes highly variable feeding relationships both spatially and temporally, making it difficult to construct accurate representations of all feeding relationships within a particular ecosystem (Winemiller & Layman, 2005). Food web models are wide-ranging, both in terms of their complexity and formulation method (McCormack et al., 2019). One approach to compile energy flow through food webs is the use of direct empirical information on consumer diets. One source of information is the analysis of stomach contents. This method, although commonly used, has its drawbacks, for example, the considerable error to estimate exact food content due to the presence of inseparable, unidentifiable and partial prey (Buckland et al., 2017). In conjunction with laboratory feeding experiments and direct observation, stomach content analysis is widely used to infer the flow of carbon among nodes and food web relationships. It is also possible to calculate trophic levels for nodes in an ecosystem, using network analysis programs, such as Ecopath (Deehr et al., 2014).

As an alternative method, stable isotope analysis emerges, rapidly becoming the primary tool to infer trophic relationships and the flow of energy in the food web (Layman et al., 2012). While stomach content analysis only provides a snapshot of recent prey items, stable isotopes provide a spatial-temporal understanding of trophic relationships between organisms, making the development and evaluation of trophic structure models more realistic (Layman et al., 2015). Stable isotope analysis made possible to estimate trophic relationships between size classes, species, and groups of species, and have been used to assess trophic levels, the ratio between the body mass of predators and preys, transfer efficiency between trophic levels, food chain lengths and the contribution of a food source to consumers diet (McCormack et al., 2019). Carbon (C) and nitrogen (N) are the most commonly used elements in stable isotope studies for marine food webs (McCormack et al., 2019). The ratio between the heavier isotope and the more common lighter isotope represents the stable isotope composition of an element in the tissue of a consumer (McCormack et al., 2019). The isotope ratio of a consumer will reflect that of its prey, depending necessarily on the isotopic ratio of the primary producers in the system (McCormack et al., 2019), allowing to determine the source of the feeding pathway, considering the fact that ratios of carbon isotopes tend to vary substantially among primary producers but change little with trophic transfers (McCormack et al., 2019). Stable isotope analysis is also useful to assess the skill, i.e. the ability to accurately predict food web characteristics, of the previous mentioned models, comparing the modelled attributes of the food web with stable isotopic composition data (Dame & Christian, 2008). The evaluation of a model skill is important to assess the accuracy and robustness of its output, to better predict ecosystem responses to threats and environmental shifts (McCormack et al., 2019).

In this dissertation, stable isotope analysis of δ^{13}C and δ^{15}N will be used to characterize the trophic web of rocky reefs of the Professor Luiz Saldanha Marine Park (Arrábida MPA), improving the understanding of the structure of this food web and its underlying ecological processes. This study will also be used to validate following studies with the ultimate objective of providing scientific advice in ecosystem-based management decisions in a rapidly changing world.

1. Introduction

The ocean is essential for human subsistence and well-being since it provides important services, such as climate regulation and food provision (Costanza, 1999). However, marine habitats have been greatly impacted by human activity and global changes (IPCC, 2019). Among marine habitats, coastal habitats, i.e., regions where the interactions between marine and terrestrial processes occur, are especially affected by human pressures. These zones attract human populations since they provide essential socio-economic value and accommodate a large variety of habitats and high levels of biodiversity (Duarte Santos, 2014). Coastal marine habitats are fundamental to coastal communities and provide several beneficial services such as nutrient cycling, pollutant detoxification, food production, raw materials, habitats, coastal protection, and recreational and entertainment activities (Foster et al., 2017). However, these services cause coastal habitats to be densely populated areas (Duarte Santos, 2014), exerting high levels of stress on them. These stressors include various factors such as pollution, overexploitation, invasive species, nutrient discharges, and sedimentation (Feist & Levin, 2016). Nevertheless, the biggest threat these stressors pose to marine coastal habitats is a change in the structure and function of these systems. All the threats mentioned above cause shifts in biodiversity (Rocha et al., 2015) with likely effects on food webs that ultimately change the structure and function of ecosystems (Crain et al., 2009), and thus the deterioration of key ecosystem processes such as the carbon and nutrient cycles (Davies et al., 2011).

Coastal marine habitats are located near to shore and include a variety of ecosystems and geological formations, from rocky shores to sand dunes, from estuaries to coral reefs (Duarte Santos, 2014). Generally, the marine coastal zone is considered to extend to a depth of 60 meters (Duarte Santos, 2014; Halpern et al., 2015). Despite their ecological importance, rocky reef habitats are one of the most impacted by anthropogenic influence (Helmuth et al., 2006). Rocky reefs are composed by formations of rocks from different origins, ranging from volcanic, sedimentary, and granites, to which marine biota can attach, forming a diverse community (Sánchez-Rodríguez et al., 2015). This habitat can reach from the bottom of the sea to sublittoral and littoral zones, where it can be exposed during low tide. It is characterized by the high spatial-temporal variability of its biodiversity, exhibiting complex communities represented by almost every taxonomic group, that depend on the environmental conditions and one another (Satyam & Thiruchitrambalam, 2018).

Rocky reefs usually border soft-bottom sandy areas. These two types of habitat support very distinct plant and animal communities (M. Martins et al., 2013), due to great differences in their structural complexity. Habitat structural complexity is crucial for ecological communities (Trebilco et al., 2015). Complex habitats offer niches and environmental resources which heightened species abundance and richness (Tews et al., 2004). In temperate rocky reefs, the presence and characteristics of the macroalgae cover and the architectural complexity, or rugosity, of the rocky substrate are foundational elements of habitat complexity. It is because of its complexity that rocky reefs are generally considered important breeding, feeding and nursery areas, i.e., environments where fish juveniles have increased chances of survival and enhanced growth (Sánchez-Rodríguez et al., 2015). These nursery habitats not only provide refuge from predation, but also rich food production.

Considering the profound importance of the ocean to humankind, it is urgent to protect its habitats. Rocky reefs, as nursery areas and biodiversity hotspots, are a priority for conservation efforts (Gladstone, 2007; Nelson & Burnside, 2019; Sánchez-Rodríguez et al., 2015). In this context, marine protected areas (MPAs) are considered effective tools for marine conservation and management. In conjunction with their benefits to marine ecology by maintaining and restoring biological diversity and

supporting fish stocks, MPAs provide several social and economic benefits (Laffoley et al., 2019). MPAs can be divided into several categories with varying degrees of protection, from no-take zones (i.e., no-take and no-go zone usually with absence of anthropogenic activities) to complementary zones, where certain human activities can take place. No-take zones are especially valuable for scientific research as ecosystem baselines (Laffoley et al., 2019). In these areas, human visitation, use, and impacts are strictly controlled and limited to secure their conservation (Laffoley et al., 2019). Since they are less affected by human threats, these areas provide an invaluable opportunity to better understand how marine ecosystems are structured and behave, allowing the improvement of conservation methods accordingly.

For a long time, most conservation research focused on the species as the study unit, assessing the impact that habitat destruction has on individual species or certain groups of species (Memmott et al., 2006). However, it is increasingly recognized that the species is not the only, or even the best, study unit in conservation biology. This is because species are connected in various ways to other species, e.g., trophic relationships. Thus, the extinction of a species can lead to secondary extinctions in complex ecological networks (Dunne et al., 2002). Ecological network analysis has been considered essential to understand biodiversity stability and dynamics (Landi et al., 2018). This systems-oriented methodology aims to holistically analyse environmental interactions used to identify properties that are otherwise not evident from direct observations (Fath et al., 2007). Network-based approaches are increasingly being used to analyse community structure since they enable to investigate questions, ranging from the species level to the community level, within a common formal mathematical framework (Delmas et al., 2019).

At local scales, biodiversity is organised into complex networks of interacting species (Rossberg, 2012), which provide the ecosystem processes which will in turn support goods and services valuable to human societies. An ecological food web is essentially a network flow model in which energy and matter flow from producer to consumer, from prey to predator. Food webs give insight into the feeding relationships in a system and provide a way of visualizing ecological systems and the trophic interactions between species or groups of similar species (Zanden et al., 2016). Understanding the complexity of natural systems and the steps needed to conserve them in a rapidly changing world is key for predicting the consequences of changes in biodiversity (Hagen et al., 2012).

Ecosystem models are wide-ranging, both in terms of their complexity and formulation method, with no fixed method for assessing the model's ability to correctly represent the characteristics of the ecosystem (McCormack et al., 2019). The most common types of quantitative models of marine food webs are size-based food web models, which represent the flow of biomass and energy through communities from smaller to larger individuals, and the species-based food web models, which assumes mass-balance over a given period of time with species aggregated into functional groups (McCormack et al., 2019). Size-based ecosystem modelling has been useful in predicting the consequences of fishing mortality and the effects of climate at community and ecosystem levels (Maury et al., 2007; Blanchard et al., 2012; Barange et al., 2014 in McCormack et al., 2019). On the other hand, species-based models are more used to predict cumulative changes and to formulate policy scenarios (Christensen & Walters, 2004 in McCormack et al., 2019).

Even though ecosystem models help to understand processes that affect ecosystems at various levels, the robustness of their results is affected by structural or process errors, assumptions about the natural dynamics and stochasticity of the system and errors in input data and parameters (McCormack et al., 2019) One approach to assess the skill of a model is to compare the modelled attributes of the food web with stable isotopic composition data.

Carbon (C) and nitrogen (N) are the most commonly used elements in stable isotope studies for marine food webs (McCormack et al., 2019). This approach is especially useful because it provides a spatial-temporal understanding of trophic relationships between organisms, making the development and evaluation of trophic structure models possible (Layman et al., 2015). They made it possible to estimate trophic relationships between size classes, species, and groups of species, and have been used to assess trophic levels, the ratio between the body masses of predator and prey, transfer efficiency between trophic levels, food chain lengths and the contribution of a food source (McCormack et al., 2019). The ratio between the heavier isotope and the more common lighter isotope represents the stable isotope composition of an element in the tissue of a consumer (McCormack et al., 2019). The isotope ratio of a consumer will reflect that of its prey, depending necessarily on the isotopic ratio of the primary producers in the system (McCormack et al., 2019), making it possible to determine the source of the feeding pathway. Due to their higher atomic mass, heavier isotopes are conserved during chemical reactions, causing what is known as trophic fractionation, and consequentially a difference between the stable isotope composition of a consumer's tissue compared to that of its prey, called isotopic enrichment (Fry, 2006; McCormack et al., 2019). The ^{15}N/^{14}N ratio is used as a marker for trophic position because δ^{15}N increases 2,5–4,5‰ from prey to predator. This isotope ratio is useful to describe the basic typology of food webs, such as food chain length and the maximum trophic position (Peterson & Fry, 1987; Post, 2002 in Layman et al., 2015; Owens, 1987 in Vinagre et al., 2015; McCormack et al., 2019). The δ^{13}C ratio can be used to determine the origin and pathways of organic matter within the food web (Layman et al., 2015; McCormack et al., 2019). This ratio is important because it allows the quantification of the pelagic and benthic contributions for the food web when the primary sources are isotopically different (Hobson et al., 2002).

Despite its importance, food web studies of temperate subtidal rocky reefs are not very abundant, even more so with stable isotope analysis. Moreover, there are still not enough studies to determine the effectiveness of the Arrábida MPA in relation to its food web and its ecological network in general. This dissertation's primary objective is the characterization of the trophic web of rocky reefs of the Professor Luiz Saldanha Marine Park (Arrábida MPA), through stable isotope analysis (δ^{13}C and δ^{15}N). To achieve this, it was determined the stable isotopic composition of species covering different trophic levels of the food web. Species were selected based on the abundance, size-based information, and other important traits, previously obtained during the ReefNets project surveys. This analysis will shed light on the connections between species and will be used to improve the understanding of the structure of these food-webs as well as the driven ecological processes. The ultimate objective of this thesis was to generate data that would be used to validate ecological network models.

2. Material and Methodology

2.1. Study area

The Professor Luiz Saldanha Marine Park (Arrábida MPA) is located on the south coast of the Setúbal Peninsula, between Figueirinha beach and Cape Espichel (Fig. 2.1). This marine park, created in 1998 and covering an area of 53 km^2, is a protected area of the Portuguese national system and part of the Natura 2000 network. It is characterized by its rocky bottom, resulting from the fragmentation of the cliffs, by its proximity to the highly productive Sado estuary and to submarine canyons. Its geographic location offers protection against northern winds, common along the Portuguese coast. These characteristics allow a variety of habitats that sustain high biodiversity, with more than 1400 marine species described (Gonçalves et al., 2015).

Arrábidas' MPA includes 4 km^2 of total protection zone (i.e. no-take zone), four partial protection zones covering 21 km^2, and three complementary protection zones with 28 km^2 in total (Henriques et al., 2013). As a result, this marine park is subject to a human pressure gradient, since in the centre (total protection zone) any human activity is prohibited, in the partial protection zones recreational activities are allowed, and in the complementary protection zones recreational fishing and commercial fishing with gillnets are allowed. Furthermore, there is also an environmental gradient caused by different water parameters and nutrient inputs from the Sado estuary.
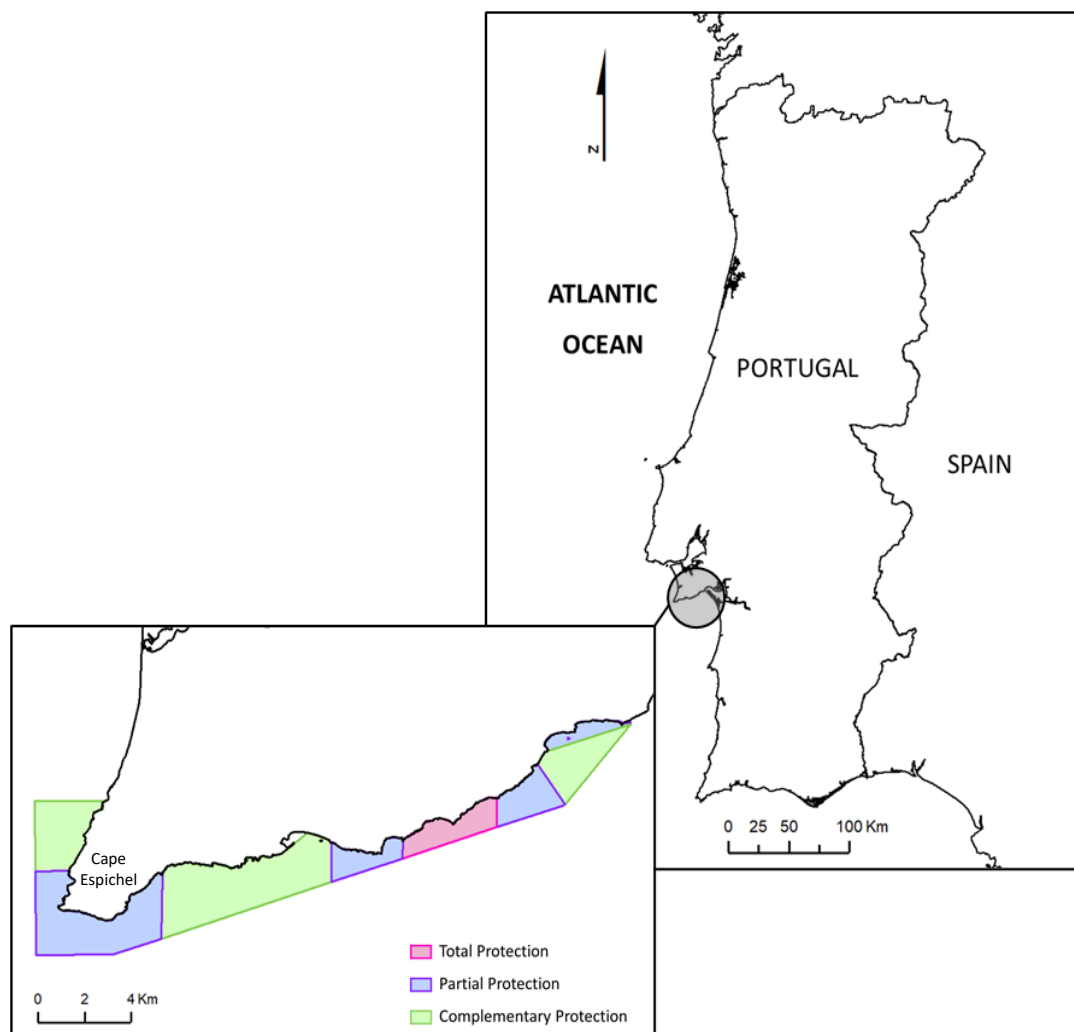

Figure 2.1. Professor Luiz Saldanha Marine Park (Arrábida MPA) location and its eight protection zones classified by level of protection: Total Protection (red), Partial Protection (blue) and Complementary Protection (green).

2.2. Sampling

The sampling took place in autumn 2020, between October and January, while scuba diving in the rocky reef of the Total Protection Area of the Professor Luiz Saldanha Marine Park (Arrábida MPA), between 5 m and 12 m deep. The total protection area was chosen because it is the baseline for the rocky reef ecosystem, with reduced human impacts and as pristine as possible, allowing the development of a base model for its food web. The samples collected were representative of the various trophic levels of the food web, namely, fishes, macroinvertebrates, microinvertebrates, macroalgae, zooplankton, phytoplankton, and particulate organic matter (POM).

The samples were collected such as to have at least three replicates, with individuals from bigger organisms counting as one replicate and smaller organisms pooled to have sufficient weight for the detection limit of the instrument for stable isotope analysis. Fish were caught with nets; macroinvertebrates were collected by hand and with nets; three octopus were caught with traps and released after the removal of one tentacle each; other invertebrates were collected through vertical and horizontal scrapings with suction pumps and nets; zooplankton was obtained through horizontal dragging with a 200 μm net, and water for phytoplankton and pelagic POM samples were collected with Van Dorn bottles at 3 to 4 meters depth. Benthic POM was acquired at the bottom, while diving, collecting the deposit that arose when rocks were raised.

2.3. Sampling processing

The samples were taken to the MARE - Marine and Environmental Sciences Centre - laboratory. Invertebrates for which it is difficult to remove muscle tissue only were visually identified, using a stereoscopic magnifying glass when necessary, and separated by class and put into several aerated aquariums filled with filtered seawater to allow gut evacuations and thus avoid trophic level errors associated with recently ingested food. After 24h organisms were retrieved from the aquariums and frozen. Invertebrates were grouped into Microinvertebrates (<3.0 cm) and Macroinvertebrates (>3.0 cm) to simplify the analysis. Fishes and larger macroinvertebrates were immediately frozen since their muscle tissue is easy to dissect. Zooplankton samples were passed through a 200 μm sieve, to remove excess water, and dried afterwards. Water for phytoplankton and POM samples was pre-filtered using a 200 μm sieve, to remove bigger particles and then passed through a 63 μm sieve. For phytoplankton samples, the residue from the 63 μm sieve was resuspended with deionized water and filtered using pre-combusted GF/C filters (47 mm; 0.7 μm nominal pore size). For POM samples, the filtrate was filtered using pre-combusted GF/F filters (47 mm; 1.2 μm nominal pore size). All filters were dried for 48 hours at 60 °C, weighed, and then frozen. Macroalgae were identified, weighed, and dried at 60 °C. After thawing, muscle tissue of fishes and muscle tissue and gonads of macroinvertebrates were removed. All samples were dried at 60 °C.

All dried samples were grinded to fine powder with a mortar and pestle and stored in labelled Eppendorf microtubes. Samples containing carbonates, such as shells, were duplicated. The replicates were engulfed in HCl 1 M, until they stopped reacting, and then centrifuged at 10000 rpm for 5 minutes, washed thrice with distilled water, and dried overnight at 60ºC. Approximately 1 mg of each animal sample and 4 mg of each plant sample were weighted in a microbalance, with an uncertainty of ± 0,001 mg, and packed in tin capsules.

2.4. Stable isotope analysis

Stable isotope ratio analysis was performed at LIE - Stable Isotopes Analysis Facility, at the Faculdade de Ciências, Universidade de Lisboa - Portugal. $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston & Owens, 1983), on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. The isotope ratios were expressed in parts per thousand (‰) according to:

$$\delta X = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 10^3,$$

where X is ^{13}C or ^{15}N, R is the ratio between the heavier isotope and the lighter one, and δ is the measure of heavy to light isotopes in the sample. $\delta^{15}N_{Air}$ values are referred to Air and $\delta^{13}C_{VPDB}$ values are referred to VPDB (Vienna Pee Dee Belemnite). The reference materials used were IAEA N1, IAEA N2, BCR-657 and IAEA-CH7 (Coleman & Meier-Augenstein, 2014); the laboratory standards used were Rice Flour, for vegetal samples, and Protein Standard OAS/Isotope (Elemental Microanalysis, UK), for animal and sediment samples. Isotope ratio analysis uncertainty per batch, calculated using 6–9 replicates of laboratory standard interspersed among samples, was < 0.15‰.

The major mass signals of N and C were used to calculate total N and C abundances, using Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.47%N, 39.53%C), for vegetal samples, and Protein Standard OAS (Elemental Microanalysis, UK, with 13.32%N, 46.5%C), for animal samples, as elemental composition reference materials.

2.5. Data analysis

Statistical analysis was carried out using Rstudio™ v.4.1.0 Software (RStudio Team, Boston, MA, USA; (RStudio Team, 2021)).

Stable isotope data was plotted in a $\delta^{13}C$ – $\delta^{15}N$ bi-plot based on mean stable isotope signatures of all taxa. Relative position of taxa in this bi-plot space was used to infer aspects of food web structure and, using the SIBER package (Jackson et al., 2011), calculate Layman's (2007) food web metrics: $\delta^{15}N$ range (dNR), a representation of vertical structure within the food web; $\delta^{13}C$ range (dCR), a metric for niche diversification at the base of the food web; total area (TA), which represents a measure of the total amount of niche space occupied; mean distance to centroid (CD), that provides a measure of the average degree of trophic diversity within the food web; mean nearest neighbour distance (MNND), a measure of the overall density of species packing; and standard deviation of nearest neighbour distance (SDNND), which measures the evenness of species packing. The SIBER package estimates food web metrics according to the distribution of values for each consumer group, using a Bayesian version of Layman's metrics, enabling the estimation of the variance on each metric and incorporate uncertainties like sample biases and small and unequal sample sizes (Jackson et al., 2011). However, TA was not used as it always increases with sample size, making the comparison between groups with unequal sample sizes impossible (Jackson et al., 2011). Instead, isotopic niches of the trophic groups were quantified based on standard ellipse areas (SEA), since they generally contain about 40% of the data and therefore are not sensitive to sample size (De Smet et al., 2015). Nonetheless, the tendency towards underestimating the SEA remains when dealing with small sample sizes, thus the small sample size-corrected SEA (SEA_C) was also calculated (Jackson et al., 2011). Results were then graphically

compared between trophic groups based on the visual analysis of the credibility intervals, where the degree of overlap between the Bayesian distributions was used as an indication of similarities/dissimilarities between groups.

Trophic levels and benthic source contribution to the diet of consumers were calculated using the Bayesian approach incorporated in the tRophicPosition R package (Quezada-Romegialli et al., 2018). This approach includes individual variability and propagates sampling error of trophic discrimination factors (TDF), i.e., the isotopic difference between two consecutive trophic levels, baselines and higher consumers in the model, and posterior estimates of parameters. An additional advantage to this approach is that it includes δ^{13}C when estimating trophic position in systems with multiple energy pathways.

The full model with the dual baseline Bayesian approach was used to discriminate two distinct sources of C and N, pelagic or benthic. This model utilizes the following equations:

$$TP = \frac{\delta^{15}N_c - \alpha\,\delta^{15}N_{b1} + (1-\alpha)\,\delta^{15}N_{b2}}{\Delta^{15}N} + \lambda$$

and

$$\alpha = \frac{\delta^{13}C_c - \delta^{13}C_{b2}}{\delta^{13}C_{b1} - \delta^{13}C_{b2}}$$

Where TP is the consumer's trophic position, $\delta^{15}N_c$ and $\delta^{13}C_c$ are the δ^{15}N and δ^{13}C of the consumer, $\delta^{15}N_{b1}$ and $\delta^{13}C_{b1}$ are the δ^{15}N and δ^{13}C of the baseline 1 (benthic baseline), $\delta^{15}N_{b2}$ and $\delta^{13}C_{b2}$ are the δ^{15}N and δ^{13}C of the baseline 2 (pelagic baseline), $\Delta^{15}N$ is the trophic enrichment factor (TEF), λ is the trophic position of the baselines and α is the contribution of baseline 1 to the diet of the consumer.

TDF was set to Post's (2002) 0.39 ± 1.3‰ for δ^{13}C and 3.4 ± 0.98‰ for δ^{15}N, which are commonly used in marine ecosystems and across a wide range of ecological studies. For the calculation of trophic position, the pelagic baseline was composed by phytoplankton and pelagic POM samples and the benthic baseline was comprised by benthic POM and macroalgae samples. The trophic level was assigned according to the calculated trophic position, as follows: 1.3-1.9 for primary consumers; 2.0-2.9 for secondary consumers; and TP over 3 for tertiary consumers. To determine benthic source contribution to the diet of secondary and tertiary consumers, the pelagic baseline was represented by zooplankton and the Polyplacophora group was considered as the benthic baseline. Using primary consumers instead of the various sources avoids the use of an undetermined mixing model, since the number of sources is greater than the number of isotopes (Cresson et al., 2020).

3. Results

3.1. General Food Web Structure

The isotopic composition of the 48 taxa analysed revealed a considerable range in both δ^{13}C and δ^{15}N values for food web components (Table 3.1, Fig. 3.1). Primary producers were the group that showed the greatest dispersion of δ^{13}C values, with mean stable carbon isotope ratios ranging from -23.8‰, for the macroalgae pool, to -12.8‰ for benthic POM. These values also represent the absolute maximum and minimum of δ^{13}C of this food web. The δ^{15}N mean values of primary producers ranged from 3.0‰ to 5.5‰, for *Treptacantha usneoides* and phytoplankton, respectively. The macroalgae *T. usneoides* had the lowest δ^{15}N value of all taxa analysed.

As for primary consumers, the mean values of δ^{13}C ranged from -22.7‰ to -15.3‰, for zooplankton and Polyplacophora, respectively, thus presenting the second highest dispersion of δ^{13}C. Mean δ^{15}N values ranged from 5.0‰, for *Sphaerechinus granularis*, to 7.0‰, for amphipods, decapods and Gorgonia. The average δ^{13}C values of secondary consumers ranged from -20.5‰, for mysids, and -16.2‰, for *Necora puber*. As for the mean values of δ^{15}N, the secondary consumer with the highest value was the fish *Parablennius gattorugine*, with 10.9‰, and the lowest value corresponds to polychaetes, with 7.4‰. This group presented the highest dispersion of δ^{15}N. Tertiary consumers were the least dispersed group, with considerable overlap, highlighting *Diplodus vulgaris* as the top consumer, for its highest value of δ^{15}N, with 12.5‰. In general, it is possible to observe a triangular shape of the distribution of δ^{13}C and δ^{15}N values (Fig. 3.1), with producers forming the base and tertiary consumers at the top. However, it is also possible to notice a higher concentration of taxa in the left side of the graph, with the δ^{13}C values of most taxa lower than -17.0‰.
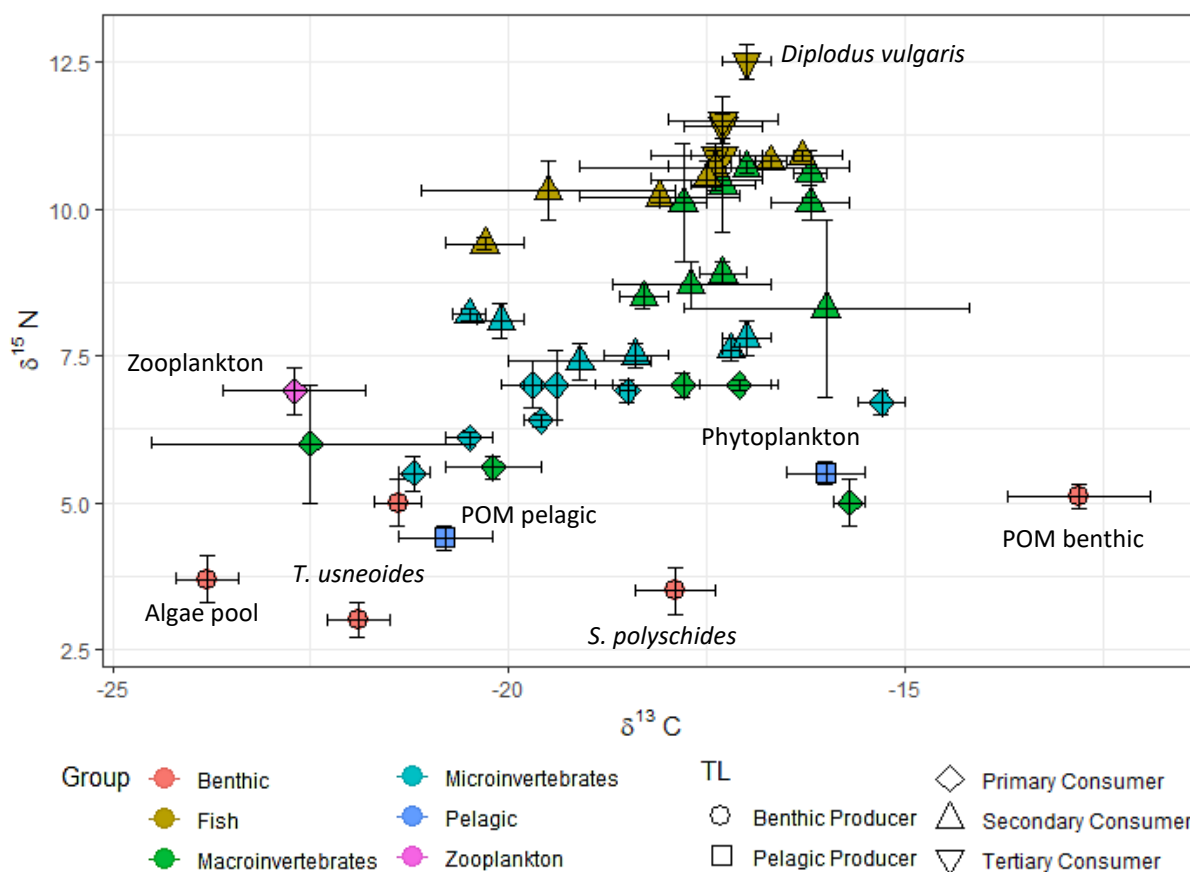

Figure 3.1. Stable isotope biplot. Mean (±SD) values of δ^{13}C and δ^{15}N for the 48 taxa sampled.

Table 3.1. Isotopic values and other metrics of the species/taxa analysed. Diet, feeding mode, trophic level, size range, number of individuals (Ind.)/pooled samples (Pool), mean δ^{13}C and δ^{15}N values and standard deviation, estimated trophic position, and trophic position retrieved from literature, with the respective references, of the 48 species/groups analysed.

Species/Taxa	Diet	Feeding Mode	Trophic Level	Size (cm)	N	$\delta^{13}C$ (%)	$\delta^{15}N$ (%)	TP_e	TP_{lit}
PRODUCERS									
POM benthic			Primary Producer		4/Pool	-12.8±0.9	5.1±0.2		
POM pelagic			Primary Producer		7/Pool	-20.8±0.6	4.4±0.2		
Phytoplankton			Primary Producer		3/Pool	-16.0±0.5	5.5±0.2		
Algae turf*			Primary Producer		3/Pool	-21.4±0.3	5.0±0.4		
Pool algae			Primary Producer		3/Pool	-23.8±0.4	3.7±0.4		
Saccorhiza polyschides			Primary Producer		3/Ind.	-17.9±0.5	3.5±0.4		
Treptacantha usneoides			Primary Producer		3/Ind.	-21.9±0.4	3.0±0.3		
MICROINVERTEBRATES									
Amphipoda*	Omnivore	Deposit-Feeder	Primary Consumer	<1.5	3/Pool	-19.7±0.1	7.0±0.4	1.9±0.4	0.8–2.7 [6]
Bivalvia	Detritivore	Filter-Feeder	Primary Consumer	<2.0	3/Pool	-19.6±0.2	6.4±0.1	1.7±0.2	1.2 [8]
Decapoda*	Omnivore	Scavenger	Primary Consumer	<2.0	3/Pool	-19.4±0.7	7.0±0.6	1.9±0.5	1.3-2.0 [5]
Gastropoda*	Omnivore	Scavenger	Primary Consumer	<3.0	3/Pool	-18.5±0.1	6.9±0.2	1.8±0.4	1.4-2.0 [8]
Hydrozoa*	Microcarnivore	Filter-Feeder	Primary Consumer	<1.5	3/Pool	-21.2±0.2	5.5±0.3	1.5±0.3	
Isopoda*	Herbivore	Grazer	Primary Consumer	<1.5	3/Pool	-20.5±0.3	6.1±0.1	1.6±0.1	
Polyplacophora*	Herbivore	Grazer	Primary Consumer	2.0-3.0	3/Pool	-15.3±0.3	6.7±0.2	1.6±0.1	3.2 [2]
Zooplankton*	Planktivore	Filter-Feeder	Primary Consumer	<0.2	3/Pool	-22.7±0.9	6.9±0.4	1.9±0.3	1.2 [8]
Crustacea*	Omnivore	Scavenger	Secondary Consumer	<2.0	3/Pool	-18.4±0.4	7.5±0.2	2.0±0.3	1.9-2.5 [5]
Felimare tricolor	Microcarnivore	Grazer	Secondary Consumer	1.5-2.5	3/Pool	-20.1±0.3	8.1±0.3	2.2±0.3	2.9 [4]
Mysida*	Omnivore	Filter-Feeder	Secondary Consumer	<1.0	3/Pool	-20.5±0.2	8.2±0.1	2.3±0.2	2.5 [6]
Nematoda	Omnivore	Predator	Secondary Consumer	<2.0	3/Pool	-17.2±0.1	7.6±0.2	2.0±0.2	1.9-3.3 [9]
Polychaeta	Omnivore	Scavenger	Secondary Consumer	1.0-3.0	3/Pool	-19.1±0.9	7.4±0.3	2.0±0.4	1.6 [7]
Sipuncula	Omnivore	Deposit-Feeder	Secondary Consumer	<2.0	3/Pool	-17.0±0.3	7.8±0.3	2.1±0.5	3.1 [2]
MACROINVERTEBRATES									
Anemonia sulcata	Omnivore	Predator	Primary Consumer	10.0-15.0	3/Ind.	-17.1±0.5	7.0±0.1	1.8±0.2	1.6 [8]
Ascidiacea	Detritivore	Filter-Feeder	Primary Consumer	5.0-10.0	3/Pool	-22.5±2.0	6.0±1.0	1.7±0.7	1.4-1.8 [5]
Gorgonia	Planktivore	Filter-Feeder	Primary Consumer	20.0-25.0	3/Ind.	-17.8±1.1	7.0±0.2	1.9±0.5	

Species/Taxa	Diet	Feeding Mode	Trophic Level	Size (cm)	N	$\delta^{13}C$ (%)	$\delta^{15}N$ (%)	TP_e	TP_{lit}
Sabellidae	Detritivore	Filter-Feeder	Primary Consumer	30.0-35.0	3/Ind.	-20.2±0.6	5.6±0.2	1.5±0.3	1.1 [5]
Sphaerechinus granularis	Omnivore	Grazer	Primary Consumer	10.0-12.0	3/Ind.	-15.7±0.2	5.0±0.4	1.3±0.6	2.0 [6]
Galathea strigosa	Detritivore	Deposit-Feeder	Secondary Consumer	8.0-9.0	3/Ind.	-17.3±0.3	8.9±0.2	2.4±0.5	1.7-2.0 [5]
Holothuria forskali	Detritivore	Deposit-Feeder	Secondary Consumer	20.0-25.0	3/Ind.	-17.7±1.0	8.7±0.4	2.3±0.5	2.0 [6]
Marthasterias glacialis	Macrocarnivore	Predator	Secondary Consumer	22.0-25.0	3/Ind.	-18.3±0.3	8.5±0.2	2.3±0.4	3.3 [6]
Necora puber	Omnivore	Predator	Secondary Consumer	5.0-7.0	3/Ind.	-16.2±0.2	10.6±0.4	2.8±0.5	2.6 [5]
Octopus vulgaris	Macrocarnivore	Predator	Secondary Consumer	>30.0	3/Ind.	-17.3±0.4	10.4±0.8	2.9±0.7	2.7-3.4 [3]
*Ophiuridea**	Omnivore	Scavenger	Secondary Consumer	10.0-15.0	3/Pool	-16.0±1.8	8.3±1.5	2.4±1.0	2.0 [6]
Palaemon sp.	Omnivore	Scavenger	Secondary Consumer	3.0-4.0	3/Ind.	-17.0±0.2	10.7±0.1	2.9±0.3	2.5 [8]
Scyllaridae sp.	Macrocarnivore	Predator	Secondary Consumer	8.0-10.0	3/Ind.	-16.2±0.5	10.1±0.3	2.7±0.4	3.9 [6]
Sepia officinalis	Macrocarnivore	Predator	Secondary Consumer	17.0-20.0	3/Ind.	-17.8±0.3	10.1±1.0	2.8±0.7	2.4 [7]
FISH									
Coris julis	Macrocarnivore	Predator	Secondary Consumer	15.0-18.0	3/Ind.	-16.7±0.2	10.8±0.1	2.9±0.2	3.2 [1]
Ctenolabrus rupestris	Microcarnivore	Predator	Secondary Consumer	6.5-9.5	3/Ind.	-19.5±1.6	10.3±0.5	2.9±0.5	3.3-3.5 [1]
Gobius xanthocephalus	Omnivore	Predator	Secondary Consumer	6.0-7.0	3/Ind.	-18.1±1.0	10.2±0.1	2.8±0.2	3.1 [1]
Parablennius gattorugine	Omnivore	Grazer	Secondary Consumer	18.0-23.5	3/Ind.	-16.3±0.5	10.9±0.1	2.9±0.2	2.9 [1]
Pomatoschistus flavescens	Planktivore	Predator	Secondary Consumer	3.0-4.0	3/Pool	-20.3±0.5	9.4±0.1	2.6±0.2	3.2 [1]
Scorpaena sp.	Macrocarnivore	Predator	Secondary Consumer	14.0-16.0	3/Ind.	-17.4±0.6	10.7±0.2	2.9±0.4	3.4 [1]
Symphodus sp.	Macrocarnivore	Predator	Secondary Consumer	12.0-15.0	3/Ind.	-17.4±1.7	10.7±0.4	2.9±0.5	2.9 [8]
Tripterygion delaisi	Macrocarnivore	Predator	Secondary Consumer	6.0-7.5	3/Ind.	-17.5±0.7	10.5±0.3	2.9±0.4	3.4 [1]
Diplodus vulgaris	Omnivore	Predator	Tertiary Consumer	8.0-16.0	3/Ind.	-17.0±0.3	12.5±0.3	3.4±0.4	3.2-3.5 [10]
Labrus bergylta	Macrocarnivore	Predator	Tertiary Consumer	28.0-38.0	3/Ind.	-17.3±0.5	11.4±0.2	3.1±0.3	3.2 [1]
Parablennius pilicornis	Omnivore	Grazer	Tertiary Consumer	6.5-7.5	3/Ind.	-17.3±0.9	10.9±0.3	3.0±0.5	3.2 [1]
Serranus cabrilla	Macrocarnivore	Predator	Tertiary Consumer	18.0-23.0	3/Ind.	-17.4±0.3	10.9±0.1	3.0±0.2	3.4 [1]
Spondyliosoma cantharus	Omnivore	Predator	Tertiary Consumer	15.0-23.0	3/Ind.	-17.3±0.7	11.5±0.4	3.2±0.5	3.3 [1]

[1] (Froese & Pauly, 2021)
[2] (Grall et al., 2006)
[3] (Hounaida et al., 2016)
[4] (Marić, 2016)
[5] (Schaal et al., 2010)
[6] (Palomares & Pauly, 2021)
[7] (Vinagre et al., 2012)
[8] (Vinagre et al., 2015)
[9] (Wu et al., 2019)

*Acidified samples

3.2. Food Web Metrics

The size of the standard ellipse area (SEA_C) of the tertiary consumers was significantly smaller than the other two groups (Primary Consumers: 5.57‰^{2}; Secondary Consumers: 5.57‰^{2}; Tertiary Consumers: 1.08‰^{2}) (Fig. 3.2.a). The δ^{15}N range (dNR) was higher for the Secondary Consumers at 3.50‰, and similar across the other two groups, being 1.97‰ for Primary Consumers, and 1.60‰ for Tertiary Consumers (Fig. 3.2.b). The δ^{13}C range (dCR) declined with the trophic group, being the highest for Primary Consumers, at 7.43‰, and lowest in the Tertiary Consumers, at 0.43‰ (Fig. 3.2.c). The mean distance to centroid (CD), where the centroid is the mean δ^{13}C and δ^{15}N value for all groups was lowest for the Tertiary Consumers at 0.45‰, and similar between the other groups (Primary Consumers: 1.91‰; Secondary Consumers: 1.67‰) (Fig. 3.2.d). The mean nearest neighbour distance (MNND) had a slight decrease trough the trophic groups (Primary Consumers: 0.77‰; Secondary Consumers: 0.48‰; Tertiary Consumers: 0.29‰) (Fig. 3.2.e), and its standard deviation (SDNND) was similar across all groups (Fig. 3.2.f). Visual analysis of the credibility intervals of the Bayesian implementation of the Layman metrics showed a high overlap in MNND and SDNND for all three trophic groups. Credibility intervals of dNR and CD overlap largely between primary and secondary consumers.
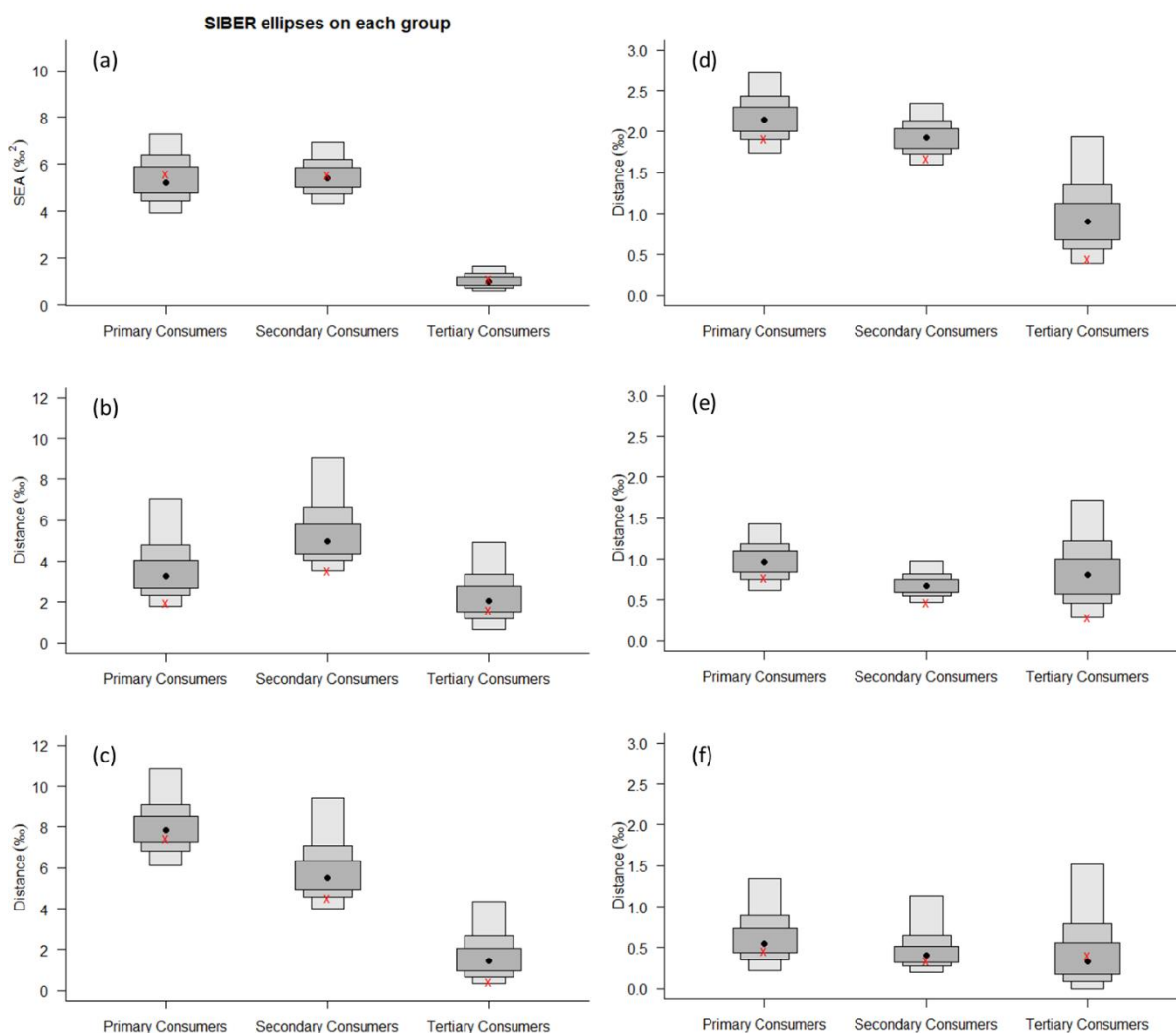

Figure 3.2. Standard ellipse areas (SEA) and Layman Metrics. Density plots of the resultant uncertainty in the SEA of consumer groups and five Layman metrics of the food web. The standard ellipse areas (SEA) are shown in (a), and the other five metrics (δ^{15}N range (dNR); δ^{13}C range (dCR); mean distance to centroid (CD); mean nearest neighbour distance (MNND); and standard deviation of nearest neighbour distance (SDNND)) are shown in (b), (c), (d), (e), and (f), respectively. Black dots represent their mode; the red crosses are the small sample size-corrected SEA (SEA_C) and true population values; and the shaded boxes correspond to the 50%, 75% and 95% credible intervals from dark to light. grey.

3.3. Trophic Position

The base of the food web was comprised of phytoplankton, benthic and pelagic POM, algae turf, pool of macroalgae, and specific macroalgae, namely *T. usneoides*, *S. polyschides* (Table 3.1, Fig. 3.1). The $\delta^{13}C$ values varied widely in the lowest trophic level of the food web, with grazers such as *S. granularis* and the group polyplacophora being enriched in $\delta^{13}C$ in comparison to zooplankton (Table 3.1, Fig. 3.1). The primary consumers, with trophic position (TP) between 1.3 and 1.9, were *S. granularis*, hydrozoans, Sabellidae, isopods, chitons (Polyplacophora), ascidians, bivalves, *Anemonia sulcata*, gastropods, amphipods, decapods, Gorgonia, and zooplankton (Table 1, Fig. 3.3). The secondary consumers, with trophic position between 2.0 and 2.9, were crustaceans, nematodes, polychaetes, sipunculids, *Felimare tricolor*, *Holothuria forskali*, *Marthasterias glacialis*, mysids, *Galathea strigosa*, ophiuroids, *Pomatoschistus flavescens*, *Scyllaridae* sp., *Gobius xanthocephalus*, *Necora puber*, *Sepia officinalis*, *Coris julis*, *Ctenolabrus rupestris*, *Octopus vulgaris*, *Palaemon* sp., *Parablennius gattorugine*, *Scorpaena* sp., and *Tripterygion delaisi* (Table 3.1, Fig. 3.3). The higher trophic positions, above 3.0, were occupied by tertiary consumers, such as *P. pilicornis*, *Serranus cabrilla*, *Labrus bergylta*, *Spondyliosoma cantharus*, and *Diplodus vulgaris* (Table 3.1, Fig. 3.3). The fish *D. vulgaris* was the top consumer with a trophic position of 3.4. Overall, the TP of fish was lower than the values found in the literature (Table 3.1). Those that most deviated from the estimated TP were *C. rupestris*, *S. cabrilla*, *Scorpaena sp.*, and *T. delaisi*. Food web length was considered to be equal to the maximum trophic position in this food web and, as such, it was estimated to be 3.4.
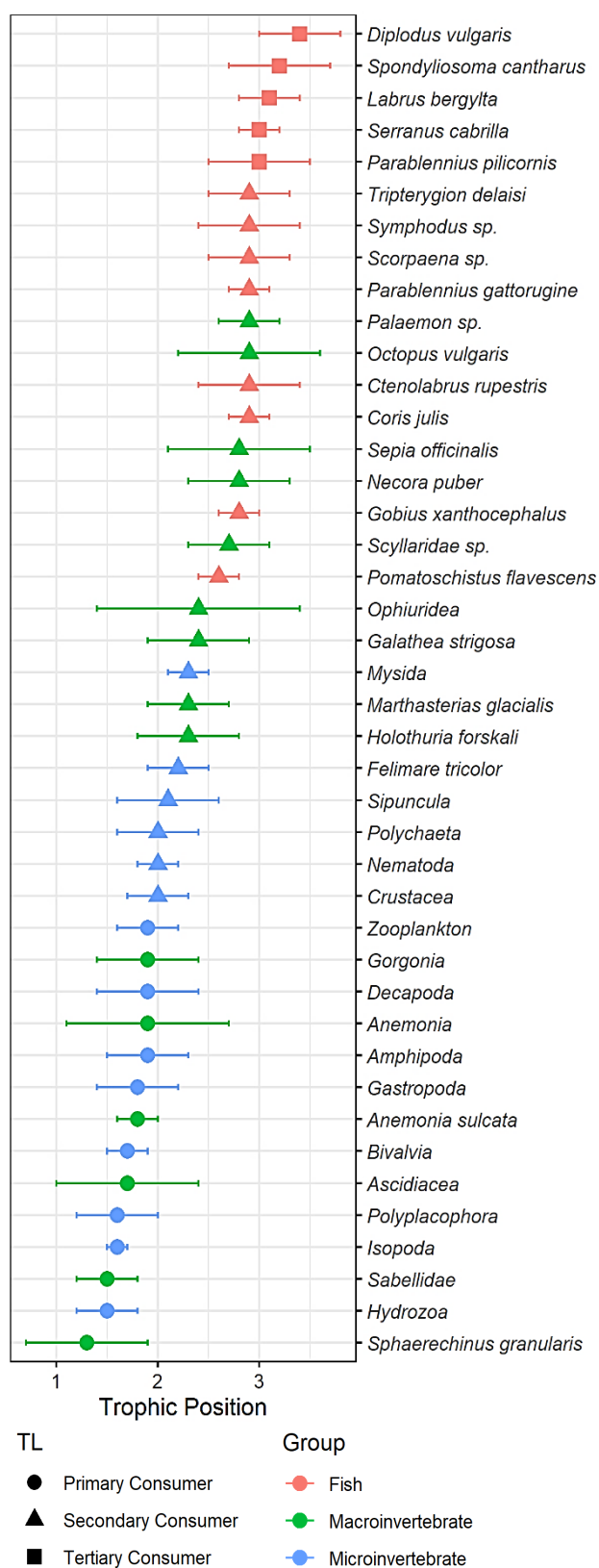

Figure 3.3. Trophic Position. Mean (±SD) trophic positions of the 41 species/groups of consumers in the food web.

3.4. Trophic Pathways

It is possible to group the sources of this food web into two groups, one based on detritus, represented in the analysis by POM, and the other based on photosynthetic production, represented by phytoplankton and macroalgae. However, the isotopic values within these two groups vary greatly, making it difficult to distinguish their relative contribution to the diet of consumers. It is also possible to group the sources into pelagic and benthic. The pelagic sources are comprised by pelagic POM and phytoplankton, whereas the benthic sources include benthic POM, and macroalgae coverage. However, isotopic values within these groups are also very dispersed, with benthic POM much more enriched in $\delta^{13}C$ than macroalgae and pelagic POM considerably depleted of ^{13}C relative to phytoplankton (Table 3.1, Fig. 3.1). Taking this into account, it is not feasible to use these sources to determine the contribution of benthic resources to the food web. To avoid this problem, secondary consumers were used as a proxy for the pelagic and benthic trophic pathways, overcoming the limitation of having more than two possible sources. There was a wide isotopic difference between pelagic consumers, namely zooplankton, and benthic consumers, such as the Polyplacophora group, indicating that stable carbon isotope analysis may be used to discern the relative contributions of these two baselines. Nevertheless, it is important to note that phytoplankton presented unexpected high values of $\delta^{13}C$, when compared to zooplankton.

Figure 3.4. Benthic source contribution. Mean ($\pm$SD) benthic source contribution to the diet of secondary and tertiary consumers.

The analysed food web showed a high dependence on the benthic pathway, with benthic sources contributing greatly to the diet of secondary and tertiary consumers (Fig. 3.4). In the group of microinvertebrates, benthic source contribution for *F. tricolor* and mysids was the lowest, at 0.3. Polychaetes had equal benthic and pelagic contribution to their diet, followed by crustaceans, with a benthic source contribution of 0.6. Nematodes and sipunculids were the most dependent of benthic sources, with a contribution of 0.7. The greatest reliance on the benthic pathway was found in the macroinvertebrate group, where benthic source contribution was the highest for *N. puber* and *Scyllaridae* sp., at 0.8, and the lowest was 0.5, for *M. glacialis*. The benthic baseline was also important for the diet of most fish, being *C. julis* and *P. gattorugine* the most reliant on benthic sources, at 0.7. *D. vulgaris*, *L. bergylta*, *P. pilicornis*, *Scorpaena sp.*, *S. cabrilla*, *S. cantharus*, *Symphodus sp.*, and *T. delaisi* had all a benthic contribution to their diets of 0.6. The fish less dependent on benthic sources was *P. flavescens*, with only 0.3, followed by *C. rupestris*, at 0.4.
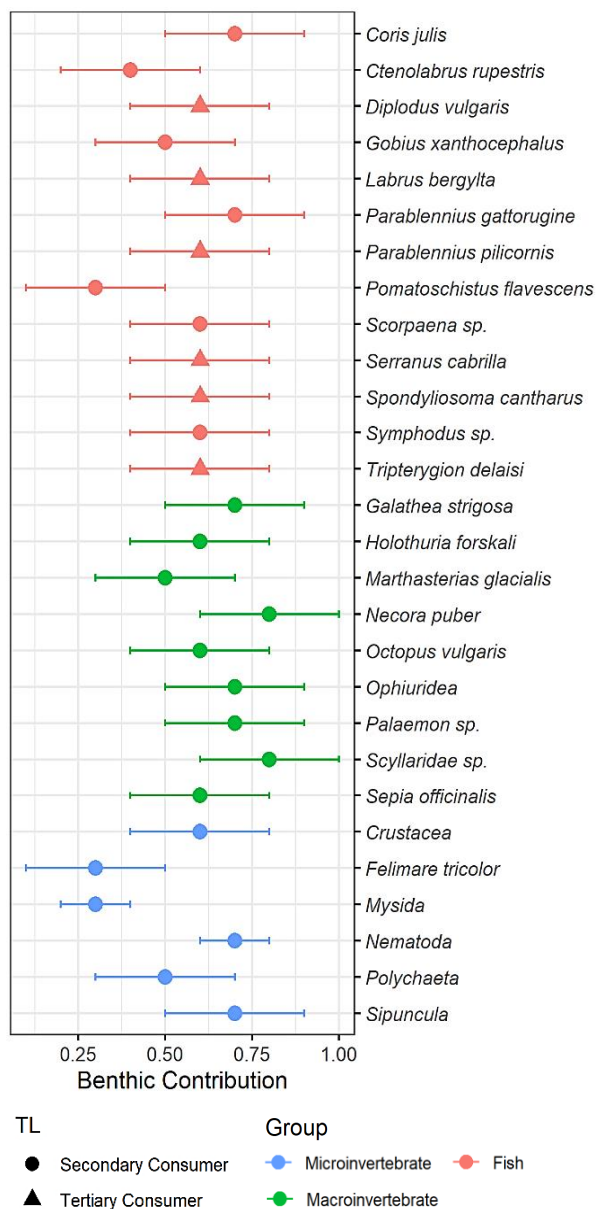
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4. Discussion

4.1. Food web structure

Stable isotope analysis of the most abundant and representative taxa in rocky reefs of the Arrábida MPA gave insight into the high complexity of its food web structure. The primary consumers analysed displayed close δ^{15}N values, while their δ^{13}C values were distributed over a wide range. This pattern seems to be the most commonly observed in temperate subtidal rocky reefs due to different types of macroalgae (Fariña et al., 2008; Fredriksen, 2003; Kang et al., 2008; Schaal et al., 2010). The wide range of δ^{13}C displayed by marine algae is influenced by the origin of the carbon atom, such as the fixation of HCO_3^{-} rather than dissolved CO^2 or the use of inorganic carbon of different origins (Raven et al., 2002 *in* Schaal et al., 2010). In this study POM was also analysed, and benthic POM increased the δ^{13}C range of the primary sources. Similar to what was reported by Vinagre et al. (2015) for the Portuguese intertidal rocky shore, but contrary to what was described by Schaal et al. (2010) in a kelp forest in northern Brittany, benthic POM showed high δ^{13}C values and was considerably enriched in δ^{13}C compared to macroalgae and pelagic POM. This can be explained by the fact that POM becomes enriched in the heavier C isotope as particles are degraded and altered by detritivores and bacteria, whilst sinking in the water column, and subsequently degraded on the ocean floor, further enriching the δ^{13}C ratio of organic matter (Nadon & Himmelman, 2006). The isotopic values of macroalgae vary with their growth cycle, which is known to be very seasonal (Schaal et al., 2010). Marine producers manifest an enrichment of δ^{13}C and δ^{15}N in summer (Ng et al., 2007; Vizzini & Mazzola, 2003), due to higher irradiance levels and seasonal differences in the utilization of ammonium and nitrate (Ng et al., 2007). POM can also vary seasonally, depending on environmental factors (Nadon & Himmelman, 2006), and its enrichment of δ^{13}C and δ^{15}N in summer has also been described in the Mediterranean, due to phytoplankton composition (Vizzini & Mazzola, 2003). As such, the higher δ^{13}C values of benthic POM, compared with pelagic POM, could also be explained by the dominance of dinoflagellates in summer and autumn (Vizzini & Mazzola, 2003). This study represents only a snapshot of the food web, and it would be interesting, in the future, to repeat the analysis in different seasons, in order to improve the understanding of the seasonal changes on this trophic web.

The δ^{13}C range (dCR) of primary consumers was large compared to the δ^{15}N range (dNR) in accordance with other studies in rocky reefs and macroalgae beds (Dyer, 2018; Heyns-Veale et al., 2019; Kang et al., 2008; Schaal et al., 2010). The wide δ^{13}C ranges for sources and consumers strongly suggest that consumers take advantage of a variety of food sources of both benthic and pelagic origin, rather than a unique one, common in pelagic ecosystems, where pelagic sources such as phytoplankton and surface POM, are the primary energy and matter contributors (Cardona et al., 2012; Kaehler & Pakhomov, 2000). In the present study, trophic groups reported to be mainly primary consumers, such as grazers and deposit-feeders, displayed close and low δ^{15}N, which is consistent with a strict primary consumer status. However, the trophic position (TP) of *H. forskali* and *G. strigosa* were slightly higher than expected, and they were classified as secondary consumers despite being detritivores. High δ^{15}N values for holothurians were observed by Nor Eddine et al. (2019) in seagrass meadows in the southwestern Mediterranean Sea. Likewise, in this study tissue from the retractor muscles was used, which is different from integument tissue, more commonly used, since the muscle does not contain carbonates. This difference in tissues implies a difference in biochemical composition but also in renewal time (Carter et al., 2019). However, although the seagrass meadows analysed by Nor Eddine et al. (2019) grow on rocky substrate, it is necessary to point out the differences between these habitats and in the sources used by these organisms. Unlike the sources analysed by Nor Eddine *et al.* (2019), the sources analysed in this study did not show high δ^{15}N values. Both *G. strigosa* and *H. forskali* feed

on organic detritus placed on the bottom (Nicol, 1931; Nor Eddine et al., 2019), thus the most likely cause for the higher trophic position obtained is their diet composition, which must have higher δ^{15}N values and thus higher proportions of organic matter from animal remains.

The length of the food web is relatively short, with a maximum estimated trophic position of 3.4 and no apex predators, with trophic positions superior to 4.0, shown by marine mammals and carnivorous fishes such as morays and congers (Froese & Pauly, 2021; Palomares & Pauly, 2021). This is similar to other findings in other rocky environments (Fredriksen, 2003; Kang et al., 2008; Schaal et al., 2010), but shorter than what can be observed in other marine ecosystems, such as offshore pelagic ecosystems (Broman et al., 1992; Jennings et al., 2002), where 4.0 is the most frequent food chain length (Vander Zanden & Fetzer, 2007). Food web length is a central characteristic of ecological communities (Post, 2002a). It modifies critical ecological functions like nutrient cycling, primary productivity, and atmospheric carbon exchange, as well as changing the organization of trophic interactions (Post, 2002a). The factors that are thought to best explain variability in food web length are ecological processes, such as the history of community organization, resource availability, habitat stability and ecosystem size, the last one appearing as the most crucial to food chain length in aquatic systems (Post, 2002a; Vander Zanden & Fetzer, 2007). In this study, the predators *Muraena helena* and *Conger conger*, marine mammals, and seabirds, which are the true top predators, were not analysed, inherently reducing the food chain length. It is also possible that the trophic fractionation is lower than the assumed 3.4 ± 0.98‰ for δ^{15}N (McCutchan Jr et al., 2003). Additionally, it is likely that most species with a TP above 2 described as carnivorous predators are in fact omnivorous in this ecosystem (Thompson et al., 2007). Consequently, the most likely explanation for the lower-than-expected trophic positions could be the fact that the individuals of some of the species collected are still juveniles. Rocky reefs are known to be important nursery areas, thus increasing the occurrence of juveniles (Cheminée et al., 2017). The diet, and in turn the degree of omnivory, of a species may vary spatially within a population (Parsons & Lebrasseur, 1970 *in* Wing & Jack, 2013), or it may vary during ontogeny, that is, during its development (Abrams, 2011). That could explain the fact that, contrary to what was expected, *Scorpaena sp.* and *S. cabrilla*, known piscivores (Froese & Pauly, 2021), were not the top predators. In fact, it has been reported that the diet of species of the genus *Scorpaena* change depending on size classes (Başçinar & Saglam, 2009), and smaller specimens prefer smaller and less mobile prey, such as small crustaceans (Rafrafi-Nouira et al., 2016). Likewise, the diet of *S. cabrilla* also changes with sexual maturity, with non-mature fish preferring Branchyura prey (Tuset et al., 1996). Another example is *C. rupestris*, whose collected individuals also had an average size smaller than the common maturation size (>11 cm) described in the bibliography (Skiftesvik et al., 2015). The juveniles of this species were described as demersal foragers of harpacticoid copepods, instead of polychaetes, hydrozoans and molluscs, more common in gut contents of adult fish (Sayer et al., 1995). One way to reduce this uncertainty would be to increase the sample sizes and increasing life stage representation as well (Potapov et al., 2019). Furthermore, most of the consumers displayed TP comprised between 2 and 3, which indicated a dominance of omnivory within this food web (Schaal et al., 2010). Food webs with more weak links are more stable and therefore more persistent than those with few strong links (Polis, 1994 and McCann & Hastings, 1997 *in* Wing & Jack, 2013). Omnivory, that is, when a consumer feeds at several trophic levels, causes a fractionation of the consumer's trophic position (Levine, 1980 *in* Wing & Jack, 2013). Food webs with more omnivory have fewer strong interactions and are less likely to have trophic cascades (Polis, 1994 *in* Wing & Jack, 2013). Because of this, complex communities with more omnivores in higher trophic positions are more stable, spatially and temporally (Polis, 1994 *in* Wing & Jack, 2013). Additional studies at different times of the year, to account for seasonality, with samples of organisms at different life stages would be important to improve our understanding of this ecosystem. Although the differences between obtained results and results described in the literature were minor,

different methodologies used (e.g., stomach content analysis and randomized resampling routines based on food items) for calculating TP, certainly affected the obtained results and should be acknowledged.

When analysing stable isotopes, it is necessary to consider the numerous biological factors that cause the natural variations of $\delta^{15}N$ and $\delta^{13}C$ values (Boecklen et al., 2011). One of these factors is related to the variability of the stable isotope composition with the life stage or sex (Quillfeldt et al., 2008). There are also differences in the isotopic composition of animal tissues, such as gonads, and exoskeletons, and in the variation in the rate of tissue turnover (Carter et al., 2019). Furthermore, there may also be seasonal variation in the values of $\delta^{13}C$ and $\delta^{15}N$, which are detected in tissues with different turnover rates (Seifert & Scheu, 2012). It is generally preferable to use muscle tissue, but because of its difficult extraction in small animals, the entire body of these organisms has been used. Lipid composition also differs in different tissues, individuals and species depending on trophic status. Lipids are considerably depleted in ^{13}C, lowering the animals' $\delta^{13}C$ values (Post et al., 2007). The chemical extraction of lipids is common; however, lipids of prey species constitute an important energy resource for consumers, and lipid extraction may bias the reconstruction of trophic links (Tarroux et al., 2010).

Results showed a considerable decrease in the standard ellipse area (SEA$_c$) and mean distance to centroid (CD) for tertiary consumers, indicating a smaller niche size in comparison with primary and secondary consumers, as expected. A larger niche, which often occupies a larger area in isospace, could be a result of species with large fluctuations in their stable isotope values, meaning more trophic diversity (Dyer, 2018). When analysing the $\delta13C$ range (dCR), primary consumers appear to have greater trophic richness related to basal resources, indicating greater diversity of carbon sources at the base. This could increase the possibility for niche differentiation and, consequently, increase taxonomic diversity (Heyns-Veale et al., 2019). Higher trophic diversity could also enhance trophic redundancy, or density of species-packing, among species (Layman et al., 2007). Nevertheless, the mean nearest neighbour distance (MNND) and standard deviation of nearest neighbour distance (SDNND) metrics were very similar among groups, suggesting similar trophic redundancy and trophic evenness between groups (Layman et al., 2007). This similarity could be due to the presence of juveniles that use the same resources and omnivores and generalists in all trophic groups of this food web. However, one of the biggest issues with Layman metrics is how sensitive they are to sample sizes (Jackson et al., 2011), and even though a Bayesian approach was used, the group of tertiary consumers was comprised of only five species, with its sample size considerably inferior to the other groups, having large posterior distributions with a high degree of uncertainty. To improve the use of community-wide metrics and understand the trophic structure of this food web, with the trophic niches of individual species, it is better to have samples with similar sizes, albeit larger, and thus increase the robustness of the analysis.

4.2. Trophic pathways

The $\delta^{13}C$ range was wide in the primary and secondary consumers, suggesting that these consumers have different diets and exploit different sources of organic matter, depending on their feeding strategy. Phytoplankton is assumed to be the main primary producer in the global ocean (Uitz et al., 2010), and most marine ecosystems are driven by the sedimentation of pelagic primary production (Cresson et al., 2020). There is evidence that depth has a significant effect on benthic-pelagic coupling, with deeper ecosystems, depending almost exclusively on pelagic production, whether through the consumption of pelagic organisms or through the consumption of sedimented organic matter of pelagic origin (Cresson et al., 2020; Witte et al., 2003).

The contribution of benthic sources to the diet of filter-feeders, namely mysids, was among the lowest, suggesting that these organisms get their nutrients via the pelagic route. Mysids are known to consume plankton, especially diatoms, dinoflagellates, and copepods (Viherluoto *et al.*, 2000). Another microinvertebrate whose diet depends on pelagic sources is *F. tricolor*. Despite being a benthic grazer, implying a greater benthic contribution to its diet, *F. tricolor* feeds almost exclusively on sponges (Debelius & Kuiter, 2007; McDonald & Nybakken, 1991), which in turn consume small organic particles, such as nanoplankton and bacteria (Ribes & Coma, 1999). Of the fish group, only *C. rupestris* and *P. flavescens* do not get most of their nutrients from benthic sources. *P. flavescens* is a specialized zooplankton feeder (Froese & Pauly, 2021; Berg, 1979 *in* Thorman, 1982) but *C. rupestris* is known to feed on bryozoans, crustaceans, and gastropods (Bauchot, 1987). One possible explanation for this result is that bryozoans, sessile suspension-feeders dependent on phytoplankton (Dunton & Schell, 1987), and gastropods such as *F. tricolor* may correspond to a greater proportion of the diet of *C. rupestris*.

Similar to findings by Vinagre *et al.* (2015) in intertidal rocky shores, the isotopic values of zooplankton differ considerably from those of phytoplankton. As zooplankton feeds directly on phytoplankton, one would expect their isotopic composition to reflect this. This difference is probably caused by the great variability of phytoplankton isotopic signatures over time, due to its high renewal time, which causes a time lag between sources and the isotopic assimilation of primary consumers (Vinagre *et al.*, 2015; Possamai & DJ, 2021). The mean $\delta^{13}C$ value of phytoplankton in this study (-16.0±0.5‰) is considerably less depleted than those described by other studies in temperate coastal waters (Dyer, 2018; Grall et al., 2006; Kang et al., 2008; Schaal et al., 2010). To account for the seasonal variability and temporal lag in isotopic ratios, it is suggested that future studies collect phytoplankton monthly, in the months before the study (Goering et al., 1990 in Kang et al., 2008). Although the isotopic values of zooplankton were similar to those described by other studies (Kang et al., 2008; Vinagre et al., 2015), it is also important to note that the isotopic signatures of zooplankton and phytoplankton will always depend on the proportion of the community composition, and the zooplankton collected was largely composed of fish eggs, which do not feed on phytoplankton, and thus, provide a possible explanation for the observed differences. Complementary community analysis of both groups could clarify some of the results from isotopic composition.

Benthic POM stable isotopic ratios were significantly enriched in ^{13}C in comparison to POM collected in the surface layer and other sources. As evidence of benthic-pelagic coupling in this ecosystem, benthic POM could be the result of the sedimentation of pelagic production, since its isotopic values were closer to phytoplankton, as it likely contributes to benthic POM composition (Vizzini & Mazzola, 2003). Deposit-feeders feed on detritus and organic matter in the sediment (Lopez & Levinton, 2011), so one would expect benthic POM to be a major contributor to their diet. In this food web, deposit-feeders, namely sipunculids, *G. strigosa* and *H. forskali*, presented mean values of $\delta^{13}C$ around -17‰, which is close to phytoplankton values and between the values of benthic POM and macroalgae. The benthic contribution to the diet of these organisms was high, in the order of 0.7, thus suggesting a mixed diet of benthic POM and macroalgae. However, in this study it was not possible to analyse biofilm and the microphytobenthos and their potential contribution as a food source, which could potentially discriminate better between both food sources and clarify some feeding relationships. Benthic POM and the microphytobenthos are important components to the diet of many benthic invertebrates in subtidal ecosystems, especially in sandy communities (Kharlamenko et al., 2008). Arrábida MPA's rocky reef is neighboured by sandy bottom habitats, which become more common at depths greater than 15 m, and it is likely that there is a high exchange of materials between these communities. Carrying out further studies linking these two distinct systems and better understanding how their food webs are connected

and affect each other, could be interesting to further our understanding of rocky reefs and their importance to adjacent ecosystems.

In shallower systems, such as coastal and estuarine ecosystems, benthic production is predominant, and benthic-pelagic coupling has a stronger influence (Cresson et al., 2020; Kopp et al., 2015). As a matter of fact, the isotopic values of pelagic POM are close to those of the macroalgae group. It has been reported that in kelp forests, under upwelling conditions, suspended POM is mainly composed of kelp detritus, instead of phytoplankton (Dyer, 2018). Arrábida MPA is affected by north-east Atlantic upwelling events (Wooster et al., 1976) and it's possible that pelagic POM composition is highly variable and dependent on these events. Additionally, pelagic POM can have different origins and may even have been brought from other places by currents and/or have been transported from land by terrestrial drainage (Vinagre *et al.*, 2015) like the Sado river. Like phytoplankton, pelagic POM is of great importance for filter-feeders, which in turn provide a very important link between the benthic and pelagic regions, by being predated by demersal organisms (Miller & Page, 2012).

Macroalgae are considered one of the major contributors to the primary production of rocky reefs (Branch, 2008; Derrien-Courtel et al., 2013; Fariña et al., 2008; Kang et al., 2008; Schaal et al., 2010; von Biela et al., 2016). In this study, special importance was given to the analysis of brown algae due to their dominance along a gradient of cover *T. usneoides – S. polyschides* (east-west gradient, respectively) at Arrábida MPA's rocky reef (Boaventura & Ré, 2001). Macroalgae present a wide dispersion in $\delta^{13}C$ values (Dauby, 1989 *in* Kang et al., 2008), with red algae tending to present more negative values and green algae less negative (Dauby, 1989 *in* Kang et al., 2008). In this study, it is also possible to see a wide dispersion of $\delta^{13}C$ values, with the algae pool showing more depleted values (-23.8±0.4‰) and *S. polyschides* less negative (-17.9±0.5‰). The more negative values of the algae pool are due to the fact that this pool had a sizeable proportion of red algae in its composition. As described by Kang *et al.* (2008) in macroalgal beds, comparing the much less negative $\delta^{13}C$ values of grazer herbivores, such as chitons and sea urchins, with those of the macroalgal pool, it is possible to infer that macroalgae with more negative values, such as red algae, contribute little to their diet. Kelps have been described as one of the main food sources for sea urchins (e.g. Norderhaug & Christie, 2009; Steneck et al., 2002) and one of the major contributors to the production of POM (Branch, 2008; Wada *et al.*, 2008), which in turn is consumed by filter-feeders (Fredriksen, 2003; Schaal et al., 2010). In addition to their importance as a food source, kelps, and other macroalgae, provide shelter from predation, suitable substrate for settlement and nursery for marine species (Asnaghi et al., 2013). The growth, reproduction and survival of macroalgae are highly dependent on temperature and light (Breeman, 1988 and Lüning, 1990 *in* Derrien-Courtel *et al.*, 2013), and in a context of climate change it is expected that the composition and abundance of macroalgae will change (Müller *et al.*, 2009), with the distribution of kelp species shifting north, and being replaced by more temperature tolerant species (Assis et al., 2016; Fernández, 2011). In fact, there has been a trend of decreasing abundance for *S. polyschides* and *Laminaria* species along the southwest coast of Portugal (Araújo et al., 2016).

Despite the importance of algal cover in this food web, there is a low number of herbivores, with omnivorous organisms being much more common. Sea urchins, such as *S. granularis*, are considered the most relevant benthic herbivores on temperate subtidal rocky reefs (Asnaghi et al., 2013; Byrnes et al., 2013; Hereu, 2006), being associated with strong trophic cascades (Asnaghi *et al.*, 2013; Guidetti, 2007). These trophic cascades are characterized by top-down control, i.e. the macroalgae thrive when predators are abundant and the density of sea urchins is low and the opposite happens when predators are removed and the urchin population grows and overeats the macroalgae, causing the formation of barren rocks dominated by coralline algae (Asnaghi et al., 2013; Guidetti, 2007). Sea urchins are preyed upon by several species of fish and invertebrates (Guidetti, 2007; Sala, 2004), but

sea breams, such as *Diplodus vulgaris* and *Diplodus sargus,* and wrasses such as *Coris julis* are described as the most efficient predators in subtidal Mediterranean rock communities (Guidetti, 2007). Both these fish include juvenile sea urchins in their diet and *D. vulgaris* can even consume adult sea urchins (Froese & Pauly, 2021; Guidetti, 2007). Of the macroalgae analysed, *S. polyschides*, although less frequent in the study site, seems to be the biggest contributor to the diet of these organisms. Nunes (2021) found lower cover of erect algae in the Complementary Protection Zone of the Arrábida MPA, where this macroalgae is dominant, associated with an increase in sea-urchin abundance. Hence one hypothesis is that the protection provided to the predators of these grazers in the no-take zone could be essential for the maintenance of *S. polyschides* in this ecosystem, avoiding its total replacement by other macroalgae, such as *T. usneoides*. The Arrábida MPA is a very interesting study site to better understand the importance of macroalgae in rocky reefs, since it presents a gradient in the variety of macroalgae, with *Cystoseira*, dominant in the zone nearest to the river, to kelp, dominant in Cape Espichel, and anthropogenic pressures along its length.

Contrary to what was described for shallow temperate reefs in southeast Australia, where phytoplankton was the most important food source for fish assemblages (Truong et al., 2017), in this study, the benthic pathway contributed to more than half of the diet of most consumers, suggesting a bottom-up control in this ecosystem, which means that the structure of this community is dependent on the benthic primary production (Menge, 1992). This fact is characteristic of rocky reefs, from the subtidal communities of the temperate coast of Chile (Fariña et al., 2008), through the rocky reefs of South Africa (Branch, 2008), to the macroalgal beds of Korea (Kang et al., 2008).

4.3. Conclusion

In summary, stable isotope analysis revealed the structure of the highly complex food web of Arrábida MPA rocky reef. This study was the first to use stable isotope analysis to try to represent the entirety of this particular ecosystem. Temperate subtidal rocky reefs are still not well understood, with relatively few recent studies concerning their food webs. In this study it was possible to conclude that consumers have diverse diets and feeding strategies, exploiting different sources of organic matter, of both benthic (macroalgae and benthic POM) and pelagic origin (phytoplankton and pelagic POM), shown by wide $\delta^{13}C$ ranges in primary and secondary consumers. However, the benthic pathway contributed to more than half to the diet of most secondary and tertiary consumers, suggesting a bottom-up control, and hinting at the importance held by macroalgae and benthic production in this ecosystem. There was also a high incidence of omnivory in all trophic groups, which could contribute to the similar trophic redundancy and trophic evenness found between groups. The importance of rocky reefs as nursery areas was also highlighted by the presence of juveniles in the analysis, which likely contributed to the lower-than-expected trophic position estimated of most tertiary and secondary consumers, especially piscivorous species, and the relative shortness of the food web. This study is an essential step to better understand this rocky reef and, as such, improve conservation and protection methods concerning this ecosystem. Nonetheless, further studies that incorporate biomass data could be important to describe this ecosystem in a more ecologically realistic way, as individuals vary greatly in their densities and biomass and are not evenly distributed across trophic levels. Future studies covering broader spatial and temporal scales and ensuring the inclusion of all different life stages are essential to fully grasp the processes that drive this food web.

General Conclusions

In this study, higher level consumers displayed lower trophic position than expected. Samples of these consumers were mainly composed by smaller specimens and juveniles. Rocky reefs are important nursery and feeding areas (Sánchez-Rodríguez et al., 2015), thus the higher abundance of juveniles is expected and may influence trophic dynamics in this food web. In fact, the food web in this study was relatively short when compared with other marine environments (see Vander Zanden & Fetzer, 2007). Diet is dependent on body size, mouth size and ontogeny (Abrams, 2011; Stoner & Livingston, 1984), and juveniles of piscivorous species prefer smaller and less mobile prey, such as small crustaceans, and have a more opportunistic diet (Rafrafi-Nouira et al., 2016; Sayer et al., 1995; Stoner & Livingston, 1984; Tuset et al., 1996). In truth, most consumers had trophic positions between 2 and 3, which could indicate a dominance of omnivory within this food web (Schaal et al., 2010). Another evidence for higher degrees of omnivory could be the similar trophic redundancy and evenness between trophic groups (Williams & Martinez, 2004). Food webs with more omnivore species have fewer strong interactions and are less likely to have trophic cascades (Polis, 1994 *in* Wing & Jack, 2013). Because of this, complex communities with more omnivores in higher trophic positions, and hence more weak links, are more spatially and temporally stable than those with a few strong links (Polis, 1994 and McCann & Hastings, 1997 *in* Wing & Jack, 2013), and possibly more resilient to environmental disturbances (Heyns-Veale et al., 2019), i.e. the ability of the food web to return to its original topology after the disturbance (McCann, 2000).

There are three types of mechanisms underlying food web resilience: nodal resilience, landscape resilience, and network resilience (see Thompson & Williams, 2017). The first refers to the resilience traits that individual nodes (populations or species) have, which gives them the ability to persist and recover when populations suffer disturbances. These traits can be very varied, including the ability to seek refuge, reproductive strategies, and other life-history traits. This type of resilience is not affected by disturbances to the taxon's resources or its consumers, being only a consequence of the traits of that taxon. Landscape resilience refers to processes that allow a population to recover from a reduction in abundance or from local extinctions, such as immigration from neighbouring populations, or reduced pressure from consumers who start to exploit other patches in the landscape. Finally, network resilience touches upon the fact that a population's interactions with its competitors, consumers, or resources facilitates resilience. That is, when the abundance of a particular taxon decreases after a disturbance, its consumer looks for alternative prey, or the resource used by the taxon responds favourably, facilitating the recovery of the affected taxon. Food webs with many weak links and high diversity will be more resilient to disturbances (Polis, 1994 and McCann & Hastings, 1997 *in* Wing & Jack, 2013). In marine food webs the most studied mechanisms are nodal resilience and network resilience. In a network resilience perspective, this study found resilience characteristics, such as high degrees of omnivory and trophic diversity at the base of the food web, in Arrábida MPA's rocky reef. However, nodal resilience was not considered, and it is thought that both contribute to the general resilience of food webs, being also dependent on each other (Thompson & Williams, 2017). For example, a generalist taxon with high nodal resilience will also have specific roles in the food web, being, for example omnivorous, which contribute to the resilience of the network. Protecting and maintaining the most productive and resilient food webs would be prudent, since they may contain species that collectively retain function, productivity and resilience into the future (Dyer, 2018).

Temperate rocky reefs are highly seasonal (Beldade et al., 2006; Schaal et al., 2010), and Arrábida MPA's rocky reef has also a strong environmental gradient, therefore, further studies including spatial and temporal variation and better representativity of different life stages are essential to fully

grasp the processes that drive this food web. Stable isotope analysis is an efficient approach for inferring the trophic structure of complex communities, considering time-efficiency and the rigour of information obtained (McCormack et al., 2019). However, describing all trophic interaction between all species of a system as diverse as this is challenging and could require thousands of individuals over time (Woodward et al., 2005). Even so, this study is a key component for the understanding of the food web dynamics of temperate rocky reefs, and it will be used in junction with other ongoing studies (i.e. project ReefNets) to comprehend the ecological network of the rocky reef of the Arrábida MPA. With this knowledge it will be possible to better predict marine ecosystem responses to human threats and environmental shifts.

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